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iPS cells Repair and Regenerate Infarcted Myocardium

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Abstract

Cardiac myocyte differentiation reported thus far is from iPS cells generated from mouse and human fibroblasts. However, there is no article on the generation of iPS cells from cardiac ventricular specific cell types such as H9c2 cells. Therefore, whether transduced H9c2 cells, originally isolated from embryonic cardiac ventricular tissue, will be able to generate iPS cells and have the potential to repair and regenerate infarcted myocardium, remains completely elusive. We transduced H9c2 cells with four stemness factors; Oct3/4, Sox2, Klf4, and c-Myc, and successfully reprogrammed them into iPS cells. These iPS cells were able to differentiate into beating cardiac myocytes and positively stained for cardiac specific sarcomeric α-actin and myosin heavy chain proteins. Following transplantation in the infarcted myocardium, there were newly differentiated cardiac myocytes and formation of gap junction proteins at 2 weeks post-MI, suggesting newly formed cardiac myocytes were integrated into the native myocardium. Furthermore, transplanted iPS cells significantly ($p<0.05$) inhibited apoptosis and fibrosis and improved cardiac function compared with MI and MI+H9c2 cell groups. Moreover, our iPS cell derived cardiac myocyte differentiation in vitro and in vivo was comparable to embryonic stem cells in the present study. In conclusions we report for the first time that we have H9c2 cell-derived iPS cells which contain the potential to differentiate into cardiac myocytes in the cell culture system and repair and regenerate infarcted myocardium with improved cardiac function in vivo.

Keywords

induced pluripotent stem cells; apoptosis; fibrosis and regenerate

Introduction

Myocardial infarction (MI) leads to heart failure implicated by complex mechanisms of cardiac myocyte cell death, hypertrophy and fibrosis[1]. Unfortunately, the common therapeutic preferences to inhibit further deterioration of MI leading to end stage heart failure are very restricted. Moreover, heart transplantation required to manage end stage heart failure patients is not readily available[2]. Therefore, new cellular therapies to repair and

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regenerate injured myocardium as well as to prevent the progression of adverse cardiac remodeling are under diligent examination. Thus far, cell transplantation in the mouse, rat, and human infarcted hearts has been studied employing a wide variety of cell types such as skeletal myoblasts, embryonic stem (ES) cells, c-kit positive cardiac stem cells, $CD45^{+ve}$ bone marrow stem cells, mesenchymal stem cells (MSC), and hematopoietic stem cells[3–6]. Indeed, adult stem cells in clinical trials have furnished significant insights into cell transplantation; however, their effectiveness is still in question[7]. In contrast, ES cells have never reached in clinical trials due to ethical and additional concerns. In general, we are still in search of optimal cell types for cardiac regeneration, repair and remodeling for transplantation in the injured myocardium. Recently identified, induced pluripotent stem (iPS) cells could be a potential cell type of choice to treat heart diseases but their basic mechanisms of cardiac repair and regeneration are largely unknown.

iPS cells generated from fibroblasts transfected with four factors (Oct3/4, Sox2, Klf4, and c-Myc; 4F-IPS cells) have the potential to differentiate into cardiac myocytes in the cell culture[8, 9]. In a recent pioneering study, Terzic and colleagues generated iPS cells with 3F (without c-MYC oncogene) and also established their potential to generate cardiac myocytes[10]. There is evidence that following transplantation in the infarcted heart, iPS cells emanated from fibroblast cells can develop into cardiac myocytes; however, there is no data available that determines the capacity to regenerate infarcted heart using iPS cells emanated from cardiac origin cell type sources such as H9c2 cells. The purpose of the present work was to generate iPS cells and to test their ability to differentiate into cardiac myocytes in the cell culture system as well as to repair (inhibit apoptosis and fibrosis) and regenerate the infarcted mouse heart.

Methods

Cell Line Maintenance

Cardiomyoblast H9c2 cells, originally isolated from embryonic heart ventricular tissue[11, 12], was ordered from American type cell culture (ATCC) and maintained in DMEM. H9c2 cells and undifferentiated mouse CGR8 ES cells expressing red fluorescence protein (RFP) were maintained on gelatin plates as we previously published[13].

Generation of Transduced Vectors and iPS cells

The cDNAs coding Oct3/4, KIf4, Sox2 and c-Myc were generated by PCR cloning using high fidelity DNA polymerase and PCR primers (Oct3/4 5'-CACCATGGCTGGACACCTGGCTTC –3' and 5'-GTTTGAATGCATGGGAGA-3', Klf4: 5'-ATGGCTGTCAGCGACGCTCT-3' and 5'-AAAGTGCCTCTTCATGTGTAAG-3', Sox2: 5'-ATGTATAACATGATGGAGACG-3' and TCACATGTGCGACAGGGGCAGTGT-3', c-Myc: 5'-TTCACCATGCCCCTCAACGTGAACTT-3' and 5'-TTTATGCACCAGAGTTCG-3') and were then cloned into vector pCR2.1 (Invitrogen, Carlsbad, CA). The cDNA sequences for KIf4, Sox2 and Oct3/4 were linked through the self-cleaving 2A sequence of foot-and-mouth disease virus (5'-AAAATTGTCGCTCCTGTCAAACAAACTCTTAACTTTGATTTAC TCAAACTGGCTGGGATGTAGAAAGCAATCCAGGTCCA-3'). The sense and antisense

oligonucleotides containing 2A sequence was ligated into vector pBluscriptll SK(−) (pBS, Stratagene, La Jolla, CA), and we generated the vector pBS-2A required for further cloning. Next, we inserted Oct3/4 and KIf4 cDNAs independently into vector pBS-2A, which generated pBS-Oct3/4-2A and pBS-KIf4-2A.. Furthermore, KIf4-2A sequence was isolated, and then inserted into pBS-Oct-3/4-2A in the same reading frame to form pBS-Oct-3/4-2A-KIf4-2A, which was then fused to the Sox2 cDNA with a stop codon in the same reading frame to form pBS-Oct-3/4-2A-KIf4-2A-Sox2. The cDNA fragment of Oct3/4-2A-KIf4-2A-Sox2 was removed from the vector and cloned into the EcoRI sites of the expression vector pCX-EGFP (obtained from Dr. Masaru Okabe, Japan). For the fourth gene c-MYC cloning, EGFP present in the Oct3/4-2A-KIf4-2A-Sox2-pCX-EGFP was replaced with c-Myc cDNA. The end product generated was an expression vector containing the four genes as mentioned above.

The H9c2 cells were cultured in 6-well plates at a density of 20,000 cells per well in DMEM containing 10% FBS. Cells were grown to 80% confluency and then transduced with the expression plasmids containing Oct-3/4, Sox2, KIf4 and c-Myc, using Lipofectamin 2000 (Invitrogen) concurring to the manufacturer's instructions. Subsequent transductions were performed on day 3 and 5, cells were incubated for seven days, and media were changed every 48 hours.

Transduced H9c2 cells converted into iPS cells were trypsinized, plated on gelatin-coated plates, and cultured in a stem cell growth medium as mentioned above. Subsequently, these cells start forming ES cell-like colonies after three weeks. We manually picked up ES cell-like colonies and cultured further on gelatin-coated plates in the ES cell culture medium and conditioned medium (CM) prepared from mouse embryonic fibroblasts (MEF) cells (50:50). Furthermore, iPS and H9c2 cells were transfected with a RFP expression vector, and stable RFP cell clones were selected with antibiotics.

Western blotting was carried out as we published[14] before to verify the expression of transduced stemness factors (4F) in the generated iPS cells from H9c2 cells. In brief, iPS or H9c2 cells (control) were lysed, centrifuged, and supernatant was isolated. Proteins were run on SDS-Page (10% polyacrylamide gel) and transferred onto PVDF membranes. After washings, incubation was performed with primary antibodies (anti-c-Myc, anti-Sox2, anti-Klf4, and anti-Oct3/4) followed by horseradish peroxidase-conjugated secondary antibodies at room temperature. After incubations, reaction was considerably matured with enhanced chemiluminescence kit (GE Life Sciences) and captured on X-ray film (Sigma, Kodak).

Determination of Pluripotency

Generated iPS cells, ES cells (positive control) or H9c2 cells (negative control) were washed 2–3 times with PBS for 5–7 min. After washings, cells were stained with alkaline phosphatase kit (Chemicon International, USA) as per manufacturer's specific recommendations. Then, cells were examined through a bright field converted microscope, and pictures were brilliantly captured.

For immunostaining, cells were fixed and permeabilized with methanol: acetone (7:3) for 20 min at −20°C, and incubated with primary antibody against Oct-3/4, a pluripotent marker

present on the ES cells. Following three washings, cells were incubated with 2° antibody Alexa Fluor 568-conjugated with IgG (Molecular Probe). Nuclei was identified with DAPI present in the mounting medium (Vector Laboratories). Cells were examined with Leica TSC SP2 laser scanning confocal microscope.

Next, we generated embryoid bodies (EBs), which contain cells from all three germ layers as reported, using our standard hanging drop method from iPS or ES cells[15, 16]. In brief, iPS or ES cells were cultured without LIF at a concentration of 2.5×10^4 cells/ml. Using our hanging drop method, 30 μl cell suspension containing 600–750 cells were developed into hanging drops in the cell culture plate for two days and then plated into in suspension culture for additional two days. Then, each EB was picked manually and placed on gelatin coated plates for up to 17 days. The generated EBs from both groups were compared to determine pluripotency as well as used for the differentiation into cardiac myocytes.

Cell transplantation in the Infarcted Mouse Heart

Animal protocols used in the current study were approved by University of Central Florida animal care committee. MI was performed as we reported[15–18]. In brief, male and female 8- to 10 week old (C57BL/6) mice were anesthetized with isoflurane, left descending coronary artery was identified and ligated with 7-0 suture as performed in our laboratory routinely. Animals demonstrating discoloration below the ligation were considered as infarcted animals and used for cell or media transplantation. Within few minutes following ligation, iPS cells (n=8), ES cells (n=8), or H9c2 cells (n=8) were transplanted by two intra-myocardial injections of 10 μl each containing 25,000 cells/injection or cell culture medium (n=8), in the peri-infarct region. After 2 weeks, heart function was determined using echocardiography as we published[15–17], and animals were sacrificed using pentobarbital (80 mg/kg, IP) injection followed by cervical dislocation. Hearts were isolated, and fixed in buffered formalin (10%) to perform further analysis.

Preparation of Tissue Sections and Histopathology

Paraffin embedded heart tissue was cut into 5 μm thick sections, deparaffinized, and rehydration was performed as we reported previously[6]. To determine cell morphology and fibrosis, sections from all heart groups were then stained with hematoxylin and eosin, and Masson's Trichrome, respectively. Cardiac fibrosis, predominantly present in the left ventricle (LV), was observed and quantified by measuring the total blue area per mm^2 with image J NIH program in the Masson's Trichrome stained sections.

Identification of Differentiated Cardiac Myocytes

Immunostainings on EBs or trypsinized beating area of the EBs were performed as we reported previously[14]. In brief, cells were fixed and blocked with 10% normal goat serum (NGS) followed by incubation with primary antibodies, anti-sarcomeric cardiac α-actin (1:100 sigma) or myosin heavy chain antibody (MHC), MF-20 (1:100, hybridoma technologies) at room temperature (RT) for 1 hour. After incubation, cells were treated with secondary antibodies, goat anti-mouse IgM or IgG (1:75, Invitrogen). Finally, sections were washed with PBS, air dried, and mounted with Anti-fade Vectashield mounting medium

containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) to stain all cellular nuclei. H9c2 cells were used as negative controls and ES cells were positive controls.

Heart sections were deparaffined, dehydrated and incubated with 10% NGS to block non-specific bindings. In brief, sections were incubated with primary antibody anti-RFP to detect donor cells; iPS cells, ES cells or H9c2 cells. Heart sections were co-labelled with primary antibody; mouse mAb anti sarcomeric α-actin (Sigma) for cardiomyocytes followed by incubations with secondary antibodies: anti-mouse or anti-rabbit Alexa 568, anti-mouse IgG conjugated with FITC, or rhodamine (M.O.M. kit, Vector Laboratories). Each set of staining includes control sections by omitting primary or secondary antibody. Sections were mounted with antifade containing DAPI to stain all nuclei, and were visualized under confocal microscope.

Apoptosis

Heart sections were deparaffinized, dehydrated and exposed to proteinase K for 20 min at room temperature for permeabilization. Commercially available apoptotic cell death detection kit was obtained from Roche Applied Bio Sciences, USA. Apoptotic staining was performed following strict instructions as explained in the flyer provided with the kit to detect apoptotic nuclei in the heart sections. We also determined amount of apoptosis by counting red apoptotic nuclei in the 1–2 heart sections from 6–8 different hearts as reported previously[6]. Apoptotic nuclei counted per section were converted into percent positive by counting the total red stained TUNEL positive nuclei divided by total blue stained DAPI positive nuclei in 4–6 randomly selected fields in the infarct and border zone area at 20×.

For caspase 3 staining, sections were co-labeled with primary antibody anti-caspase 3 rabbit polyclonal (1:50 dilution, Santa Cruz Biotechnology and cell signaling) and sarcomeric cardiac α-actin mouse monoclonal antibody (1:20; Sigma) for 1 hour at 37°C in a humidified chamber. Three washings were given and sections were incubated with anti-rabbit Alexa 635 or anti-mouse Alexa 488 secondary antibodies (Molecular Probes). Sections were mounted with Antifade medium containing DAPI (Vector Laboratories) to stain nuclei. Sections were examined with a confocal microscope.

Caspase-3 activity was performed using Bio-vision colorimetric assay as we reported previously[17].

Data Analysis

Group data was presented as means±SE. One way ANOVA followed by Tukey test to assess the significant difference between groups, $p<0.05$.

Results

Cardiac iPS cells and Their Pluripotency

Figure 1, panel A, shows H9c2 cells are elongated (left panel), whereas following transduction with 4F expression vector, these cells start appearing morphologically distinct (right panel) compared with untransduced H9c2 cells. Moreover, transduced H9c2 cell morphology appeared to be similar to growing mouse ES cells[19]. The efficiency of cluster

formation varied from .008–.01% in H9c2 cells following transduction. Subsequently, growing clones were monitored, manually picked, and maintained in cell culture medium containing MEF conditioned medium. To determine whether these four factors are present in the generated iPS cells, our western blot analysis data shows expression for these four stemness factors in the iPS cells, whereas no such factor was present in the H9c2 cells (Figure 1, panel B). Next, we determined whether generated iPS cells also express pluripotency markers such as Alkaline phosphatase and Oct3/4. As expected, iPS cells were expressing alkaline phosphatase in 100% of growing cells, whereas similar expression was observed in ES cells used as a positive control (Figure 1, panel C). In contrast, H9c2 cells were negative for alkaline phosphatase staining (Figure 1, panel C). Moreover, growing iPS cells were positive for the undifferentiated pluripotent marker Oct3/4, which was compared with ES cells as a positive control (Figure 1 panel D). These data suggest that generated iPS cells are reprogrammed, express pluripotent markers, and comparable to ES cells.

Cardiac Myocyte Differentiation From iPS Cells in Cell Culture

To test whether iPS cells have the potential to generate EBs which contain cells from all 3 germ layers as observed with ES cells, we generated EBs using our standard hanging drop method. iPS cell-generated EBs were morphologically and functionally similar to ES cell derived EBs (data not shown). Next, we determined EBs potential to generate cardiac myocytes at day 11. We observed 30% of spontaneously beating EBs generated from iPS cells compared with 27% from ES cells, suggesting the presence of cardiac myocytes with similar potential in both cell types (data not shown). Furthermore, double immunolabeling for the cardiac-specific marker, sarcomeric α-actin, and reporter protein RFP were performed to determine the presence of cardiac myocytes. Certain EBs generated from iPS cells or ES cells were highly positive for sarcomeric α-actin demonstrating the presence of cardiac myocytes (sarcomeric α-actin, Figure 2 panel A; a, e and i) and reporter protein RFP (Figure 2 panel A; b, f and j). DAPI stained total nuclei (Figure 2 panel A; c, g and k), and merged images confirm cardiac myocytes differentiation (Figure 2 panel A; d, h and l). Next, we trypsinized the beating EBs and stained with sarcomeric α-actin as shown in Figure 2, panel B. Moreover, digested EBs were also stained with another cardiac myocyte specific marker, MHC antibody, MF-20, in iPS and ES-EBs (Figure 3, panel A; e and i) whereas H9c2 cells were negative for MF-20 (Fig. 3 panel A; a). Our quantitative data suggest that there were 25% differentiated cardiac myocytes in the EBs generated from both iPS and ES cells compared with 0% in H9c2 cells (Figure 3, panel B). Therefore, we suggest that the iPS cell line has the potential to differentiate into cardiac myocytes in cell culture.

Transplanted iPS Cells Differentiate into Cardiac Myocytes and Form Gap Junctions

Heart sections stained with anti-RFP antibody demonstrated that the transplanted iPS cells were present in the peri-infarct and infarct regions of the myocardium in C57BL/6 mice (Figure 4, panel A; n) whereas there were no stained cells in H9c2 cell transplanted hearts (Figure 4, panel A; f). Next, MI+ES cell transplanted hearts stained with anti-RFP antibody demonstrated patchy areas of engrafted cells in the peri-infarct and infarct region of the heart (Figure 4, panel A; j). To confirm whether transplanted cells differentiated into cardiac myocytes, all four groups were co-labeled with sarcomeric α-actin antibody, suggetsing transplanted stem cells differentiated into new cardiac myocytes (Figure 4, panel A; a, e, i,

m). However, newly generated cardiac myocytes were rarely found in the H9c2 cell transplanted group. Quantitative data shows that there were 1.92±0.3% newly formed cardiac myocytes in ES cell transplanted hearts compared with 2.37±0.3% in iPS cells (Figure 4, panel B). The percent positive differentiated cardiac myocytes in iPS and ES groups were significantly ($p<0.05$) different compared with H9c2 cells and MI groups. However, there was no statistical difference between iPS and ES cell groups (Figure 4, panel B). Next, to confirm whether newly generated cardiac myocytes demonstrate electrical coupling in the native myocardium, heart sections were triple immunolabeled; RFP to detect donor iPS cells, sarcomeric α-actin, to detect cardiac myocytes and connexin-43, to identify gap junctions. Figure 4, panel C, shows newly generated cardiac myocytes form gap junctions, were electrically coupled, and integrated into the myocardium.

Transplanted iPS Cells Inhibit Apoptosis

Effects of transplanted iPS cells on cardiac apoptosis were determined by TUNEL staining, caspase-3 immunostaining, and caspase-3 activity. TUNEL staining data shows the reduced amount of red apoptotic nuclei in MI+iPS or ES cell groups compared with wide spread red apoptotic nuclei in MI and MI+H9c2 cell groups (Figure 5, panel A). DAPI stained total nuclei (Figure 5, panel A; b, e, h and k) and merged images are visible in c, f, i and l. Quantitative TUNEL apoptotic nuclei shows transplanted iPS cells significantly ($p<0.05$) reduce apoptotic nuclei (0.6±.07%) compared with MI or MI+H9c2 cell groups (MI; 1.2±0.1, MI±H9c2 cells; 1±0.2% apoptotic nuclei/section, Figure 5, panel C). Moreover, transplanted ES cells reduced apoptosis comparable with the iPS cell group. TUNEL stained heart sections were also combined with caspase-3 staining to confirm cell death is apoptotic in nature. Furthermore, some of the sections were also co-stained with a third antibody, sarcomeric α-actin, to confirm apoptosis occurs in the cardiac myocytes. Our data in Figure 5, panel B, confirms apoptotic nuclei were stained with TUNEL, co-labeled with caspase-3 antibody, and also positive with sarcomeric α-actin, suggesting apoptosis occurs in cardiac myocytes. Next, we measured caspase-3 activity, which is considered a hallmark of apoptosis. Our data shows that following iPS or ES cell transplantation, caspase-3 activity was significantly ($p<0.05$) reduced compared with MI, whereas H9c2 cells demonstrated no decrease in caspase-3 activity (Figure 5, panel D). Moreover, our caspase-3 activity data corroborate with the TUNEL apoptotic nuclei staining confirming apoptosis in the heart.

Transplanted iPS cells Inhibit Fibrosis

Histological examinations were performed to determine whether transplanted iPS or ES cells inhibit adverse cardiac remodeling. LV remodeling was determined by measuring total interstitial fibrotic area in mm^2 in all mice groups transplanted with or without stem cells. Heart sections obtained from infarcted hearts transplanted with iPS and ES cells show significant ($p<0.05$) decrease in fibrosis (MI+iPS cells:0.25±0.09 and MI+ES cells: 0.21±.05 mm2) compared with large fibrotic areas in MI and H9c2 cell transplanted hearts (MI: 0.94±0.03 and MI+H9c2 cells: 0.9±07 mm2, Figure 6, panel B).

Teratoma Formation

All animals transplanted with iPS and ES cells following MI demonstrated no evidence of teratoma formation as examined in the H&E stained sections (data not shown).

Transplanted iPS Cells Improve Cardiac Function

Echocardiography performed at 2 weeks after the LAD ligation showed significant improvement in cardiac fractional shortening in iPS transplanted hearts (MI+iPS cells; 45.4±0.7) compared with MI+ES cells, MI+H9c2 cells and MI (MI+ES cells; 41±0.9, MI +H9c2 cells; 38±0.7 and MI; 31±0.9, Figure 6, panel C) groups. Moreover, ES and H9c2 cell transplanted groups were significantly ($p<0.05$) different compared with MI hearts (Figure 6, panel C). Next, left ventricular interior diameter systolically (LVIDs) was significantly ($p<0.05$) decreased in MI+iPS and ES cell groups compared with MI and MI +H9c2 cell groups (Figure 6, panel D).

Discussion

The major aim of the present study was to generate H9c2 cells induced iPS cells and to establish their potential to repair and regenerate infarcted myocardium. This study is the first to successfully create iPS cells from H9c2 cells using four stemness factors (Oct3/4, Sox2, Klf4, and c-Myc). These iPS cells exhibit numerous similar characteristics of ES cells, including; formation of ES cell like colonies, presence of four stemness factors, positive staining for alkaline phosphatase and Oct3/4 as well as their characteristics to form EBs. Our iPS cell data corroborate with the undifferentiated characteristics of ES cells and iPS cells generated from human embryonic and adult fibroblasts cells[9, 20, 21]. Moreover, our successful reprogramming of H9c2 cells demonstrate a possibility to generate iPS cells from human adult or neonatal isolated cardiac myocytes.

Next, the procured data on cardiac myocyte derivation from human or mouse iPS cells in vitro and in vivo is from iPS cells transduced from fibroblasts using four stemness factors (Oct3/4, Sox2, Klf4, and c-Myc) or without c-Myc [8, 22, 23]. Our present study was designed to determine whether H9c2 cells induced iPS cells can differentiate into cardiac myocytes in the cell culture as well as repair and regenerate the infarcted mouse heart following transplantation. In this regard, we developed EBs and conclusively established that cardiac myocytes derived from iPS cells are synchronously beating and stained positive with the cardiac myocyte specific markers sarcomeric α-actin, and MHC antibody, MF-20. The current iPS derived cardiac myocyte differentiation data was compared and confirmed with a well-established cardiomyogenesis model of ES cells[14]. Moreover, recently published, mouse and human fibroblast-iPS cell derived cardiac myocytes in the cell culture system is well in accordance with the data obtained in the present study, strongly suggesting iPS cells have potential to differentiate into cardiac myocytes[8, 22, 23]. However, our present study opens new avenues to generate iPS cells from tissue specific iPS cell types compared with general fibroblast iPS cells. Whether generation of tissue specific cell types is advantageous needs further investigation.

Next, we would like to authenticate whether transplanted iPS cells have the potential to regenerate infarcted myocardium. Our data strongly suggest that iPS cells, when inserted into the infarcted heart, lose their pluripotency and engraft into the native myocardium where the local microenvironment direct them to differentiate into cardiac myocytes. Moreover, major primary concerns in stem cell transplantation studies include whether newly differentiated cells can integrate into host myocardium. Our data suggest that newly

formed cardiac myocytes are positive for connexin-43 and demonstrate electrical coupling. These heart regeneration data using cardiac iPS cells are important because iPS cells can easily be generated from various patient specific adult cell sources such as human cardiac myocytes[10, 15, 24]. Moreover, our transplanted iPS cell regeneration data is agreeable with the performed studies in the infarcted heart using ES or fibroblast-iPS cells. Moreover, transplanted H9c2 cells were unable to demonstrate new cardiac myocyte differentiation. These data suggest that transduced iPS cells have lost parental characteristics of H9c2 cells and contain better potential to regenerate infarcted heart compared with non-transduced H9c2 cells.

Transplanted ES or iPS cells can form teratomas following transplantation into immune-deficient mice, which is a well-established characteristic of ES cells[21, 25]. Accordingly, teratoma formation is a major limitation following transplantation of ES cells or iPS cells in any organ. This has recently been reported that transplanted iPS cells in the infarcted myocardium of immunodeficient mice shows tumor formation compared with immunocompetent mice where no teratoma formation was observed[10]. In the present study, we firmly suggest that transplanted iPS cells in the infarcted mouse heart can successfully engraft and differentiate into cardiac myocytes with no evidence of teratoma formation. The exact reason for the absence of teratoma formation is completely unknown. However, it is possible that we transplanted a low number of cardiac iPS cells, giving the cells more exposure to cardiac released factors following MI, which may help direct differentiation into cardiac cell types. In contrast, large numbers of transplanted ES or iPS cells will form cellular clumps, which may expose them to an internal stem cell microenvironment which drives stem cell proliferation rather than cardiac cell type differentiation, leading to the formation of teratomas. Another, whether transduced oncogene c-Myc may have lost its characteristics to form teratoma in these iPS cells remains unknown. Our data is in an agreement with previously published studies suggesting a low number of ES or iPS cells does not form teratomas[10, 26, 27].

Next, we observed significantly improved cardiac function in iPS cell transplanted hearts compared with MI controls. However, observed improved function does not correlate well with the number of newly differentiated cardiac myocytes. In this regard, we extended this study to examine whether transplanted iPS cells inhibit cardiac myocyte apoptosis and fibrosis. This is well established that apoptosis is a programmed cell death which plays a significant role in the progression of MI leading to end stage heart failure[1]. Moreover, adverse cardiac remodeling involves fibroblast proliferation, which replaces dead apoptotic cardiac myocytes resulting in the development of fibrosis, which ultimately leads to heart dysfunction[1]. In the present study, we suggest that transplanted iPS cells significantly inhibit endogenous cardiac apoptosis and fibrosis in the infarcted heart. The inhibition of apoptosis and fibrosis in the present study seems to parallel the effects observed in stem cell studies previously[6, 17, 28]. This data suggests that a significant decrease in apoptosis and fibrosis in the MI is a common phenomenon by which cell transplantation in the infarcted heart utilizes its beneficial effects mediated through autocrine or paracrine pathways. Therefore, we postulate that transplanted iPS cells also release autocrine or paracrine factors, which play a major role in the inhibition of apoptosis and fibrosis, which remains to be determined. Moreover, factors released from ES cells or MSC inhibit apoptosis[13, 18, 29, 30]. However, the

released factors are a distinct type of growth factor as reported[13, 18, 29, 30]. Similarly, to determine whether factors released by iPS cells are the same as ES cells or MSC needs further investigation. Overall, we suggest that improved cardiac function in the present study is due to the formation of new cardiac myocytes as well as inhibition of adverse cardiac remodeling.

In conclusion, we report for the first time that H9c2 cells induced iPS cells are pluripotent, can differentiate into cardiac myocytes in the cell culture system, and regenerate infarcted myocardium. We also provide the findings for the first time that two weeks post-MI iPS cells transplanted in the infarcted heart inhibit apoptosis and fibrosis. Multifactorial effects of transplanted iPS cells lead to new cardiac myocyte differentiation and a reduction in cardiac remodeling contributing to improved cardiac function.

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Abbreviations

4F	4 stemness factors (c-Myc, Oct3/4, Klf4 and Sox2)
ATCC	American type cell culture
CM	conditioned medium
DAPI	4',6-diamidino-2-phenylindole
EB	embryoid body
ES	embryonic stem
FS	fractional shortening
IP	intraperitoneal
iPS	induced pluripotent stem
LAD	left anterior descending artery
LIF	leukemia inhibitory factor
LV	left ventricle
LVIDs	left ventricular interior diameter systolically
MEF	mouse embryonic fibroblasts
MHC	major histocompatibility complex
MI	myocardial infarction

MSC	mesenchymal stem cell
NGS	normal goat serum
PBS	phosphate buffered saline
RFP	red fluorescent protein
RT	room temperature
TUNEL	Terminal deoxynucleotide transferase dUTP nick end labeling

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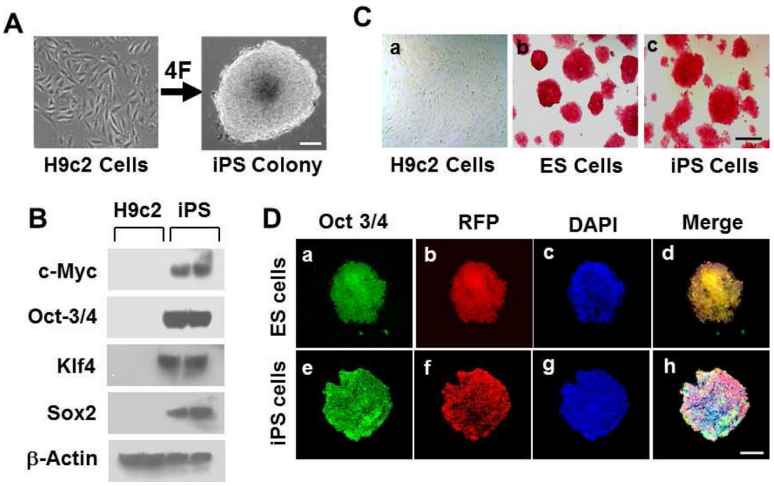

Figure 1.

Left Panel A shows photomicrographs of untransduced H9c2 cells whereas right panel shows transduced H9c2 cells forming ES cell like colony. Western blot analysis in panel B, shows no expression of 4F (c-Myc, Oct3/4, Klf4 and Sox2) in H9c2 cells, and increased expression of 4Fs was present in transduced H9c2. Scale bar = 50 μm. Photomicrographs of alkaline phsophatase shows no positive staining in H9c2 cells (panel C-a), and stained ES cells (pinkish red, panel C- b, positive control) and stained iPS cells (panel C-c). Scale bar = 250 μm. Confocal images demonstrate oct3/4 in ES cells and iPS cells (panel D-a and e), RFP-expressing ES cells and iPS cells (panel D-b and f), DAPI (panel D-c and g), and merge (panel D-d, and h). Scale bar = 100 μm.

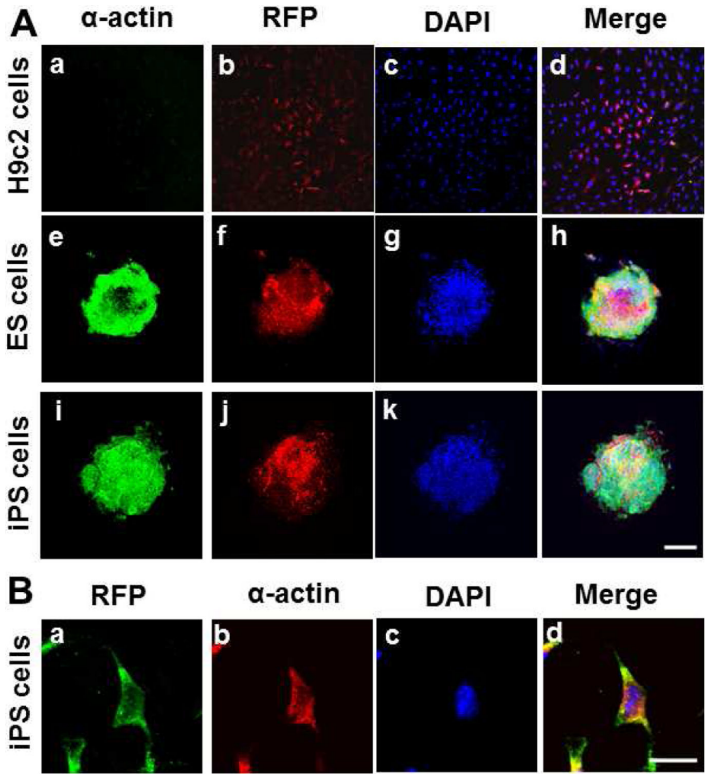

Figure 2.

iPS cells form embryoid bodies (EBs) and differentiate into cardiomyocytes. EBs were differentiated for 17 days. Some of the small EBs showed large positive areas with anti-sarcomeric α-actin/Alexa 488 (panel A-e, ES cells-EBs, and panel A-i, iPS cells-EBs), and DAPI (c, g, k). H9c2 cells did not show α-actin staining (panel A-a). The merged images (panel A-d, h, and l) shows co-expression of α-actin and RFP. Scale bar = 100 μm. Panel B shows trypsinized iPS cells stained for cardiac myocytes with anti-sarcomeric a-actin/Alexa 488, DAPI, and merge. Scale bar = 10 μm.

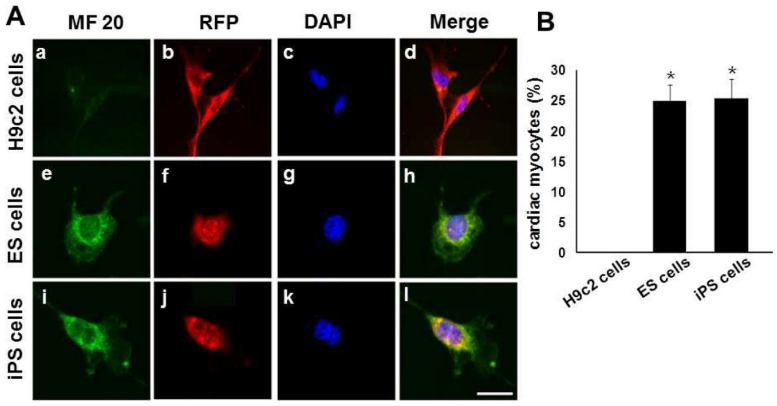

Figure 3.

iPS cells formed embryoid bodies (EBs) were trypsinized and stained with cardiac myocyte specific MHC, MF-20 (panel A-e, ES cells-EBs, and panel A-i, iPS cells-EBs), and DAPI (c, g, k). H9c2 cells did not show MF-20 staining (panel A-a). The merged images (panel A-d, h, and l) shows co-expression of MF-20, RFP and DAPI. Scale bar = 10 μm. Panel B, shows quantitative analysis of percentage positive cardiac myocytes in trypsinized EBs derived from ES and iPS cells, *$p<0.001$ vs H9c2 cells.

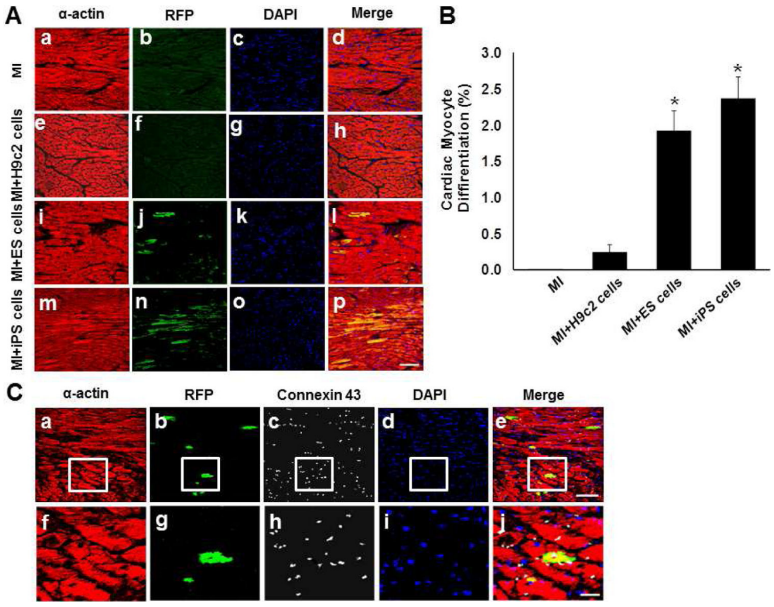

Figure 4.

Transplanted RFP-iPS or ES cells differentiate into cardiac myocytes post-MI. Cardiac myocyte specific anti-sarcomeric α-actin stained red shows cardiac myocytes (panel A-a, e, i and m), anti-RFP to identify donor cells (panel A-b, f, j and n), and DAPI (panel A-c, g, k and o). Merged images of all three stainings are shown in (panel A-d, h, l and p). Scale bar = 50 αm. Panel B, shows quantitative analysis of newly differentiated cardiac myocytes following transplantation at 2 weeks post-MI, *p<0.001 vs MI and H9c2 cells. Panel C, shows anti-sarcomeric α-actin in red (panel C-a, and f), anti-RFP (panel C-b, and g), connexin-43 to identify formation of gap junction between donor derived cardiac myocytes and native myocardium (panel C-c, and h), and DAPI (panel C-d, and i). Merged images are shown in (panel C-e and j). Panel C, top level; a-e, scale bar = 50 αm, and lower level f-j, scale bar = 20 αm.

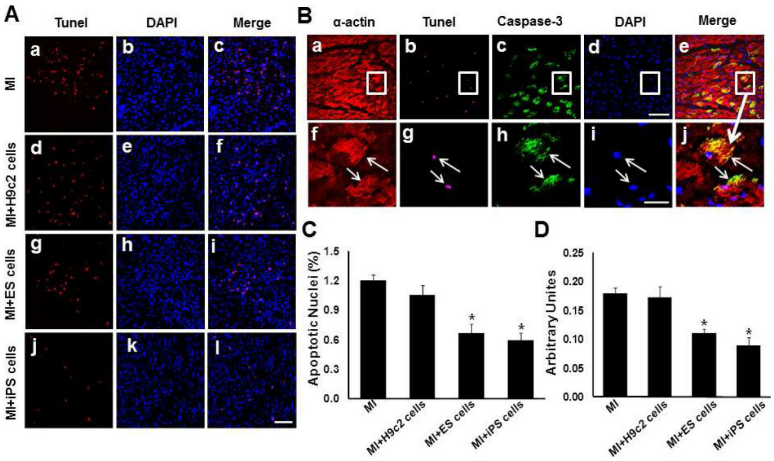

Figure 5.

Tranplanted iPS cells inhibit apoptosis at 2-weeks post-MI. Panel A shows representative photomicrographs of TUNEL stained apoptotic nuclei in red (panel A-a, d, g and j), total nuclei stained with DAPI in blue (panel A-b, e, h and k) and merged nuclei in pink (panel A-c, f, i and l). Scale bar = 50 μm. Panel C, histogram shows quantitative percentage of apoptotic nuclei. *p<0.05 vs MI and H9c2 cells. Panel B, shows anti-sarcomeric α-actin in red (panel B-a, and f), TUNEL (panel B-b, and g), caspase-3 immunolabeling, (panel B-c, and h), and DAPI (panel A-d, and i). Merged images are shown in (panel A-e and j). Panel C, top level; a-e, scale bar = 50 μm, and lower level f-j, scale bar = 20 μm. Panel D, histogram shows quantitative caspase-3 activity. *p<0.05 vs MI and H9c2 cells.

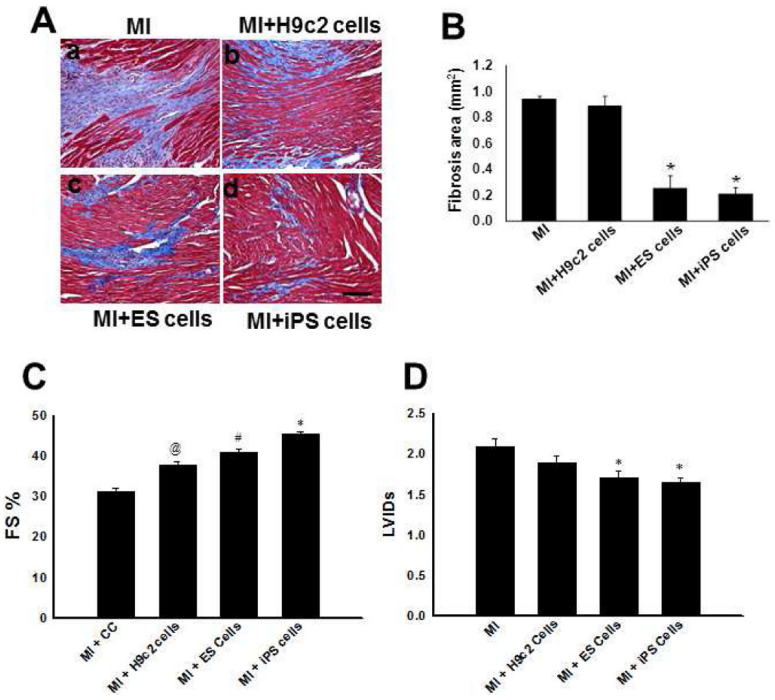

Figure 6.

Panel A demonstrates representative photomicrographs from Masson's trichrome stained heart sections with and without iPS cells transplantation post-MI; MI+cell culture medium (panel A-a), MI+H9c2 cells (panel A-b), MI+ES cells (panel A-c), MI+iPS cells (panel A-d) (Scale bar = 50 μm). Panel B, histogram shows quantitavily less fibrosis in MI+iPS or ES cell groups compare with MI+cell culture medium and MI+H9c2 cells groups (*p<0.05). Panel C, histogram shows transplanted iPS cells improves cardiac function. Average echocardiographic fractional shortening (FS) for treatment groups. *p<0.05 vs MI, H9c2 and ES cells, # p<0.05 vs MI and H9c2 cells, @p<0.05 vs MI. Panel D, Left ventricular interior diastolic diameter systolically (LVIDs) for different treatment groups. *p<0.05 vs MI, and &p=Non-significant.